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# Identification of $\beta$ -carotene oxidation products produced by bleaching clay using UPLC-ESI-MS/MS

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## ABSTRACT

The removal of plant pigments such as  $\beta$ -carotene is an aspect of vegetable oil processing often desired by the food and pharmaceutical industries. Adsorption of  $\beta$ -carotene to acid-activated clay (AAC) is a well-established method for purification. Despite this, the removal mechanism of  $\beta$ -carotene is not well understood. UPLC-MS/MS analysis of surface compounds extracted from  $\beta$ -carotene-AAC (BC-AAC) complexes show that AAC acts as an oxidiser. Oxidation products detected included canthaxanthin and 3',4'-didehydro- $\beta$ -caroten-4-one. AAC had surface water exchanged with an  $^{18}\text{O}$  labelled water and was then exposed to  $\beta$ -carotene. Carotenoids labelled with  $^{18}\text{O}$  were produced from this reaction, suggesting surface water is necessary for  $\beta$ -carotene removal.

## 1. Introduction

$\beta$ -Carotene is a bright orange pigment produced by a range of plants and microorganisms. It is a tetraterpenoid polyene containing 11 conjugated carbon-carbon double bonds. This extensive conjugation is responsible for the compound's intense absorption of visible light and its antioxidant properties. It is the parent structure for a class of compounds known as carotenoids. These can be divided into two sub-classes: carotenes and xanthophylls. Carotenes are hydrocarbon carotenoids such as  $\beta$ -carotene and lycopene. Xanthophylls are oxygen containing carotenoids such as lutein, canthaxanthin and astaxanthin.  $\beta$ -Carotene is lipid soluble and is the most abundant carotenoid in plants. Hence, it is present in most vegetable oils where it can impart a yellow-orange colour. While having health benefitting antioxidant properties,  $\beta$ -carotene and certain other carotenoids can exhibit pro-oxidant behaviour under the elevated temperatures often used during vegetable oil processing (Zeb & Murkovic, 2011, 2013). Removal of this compound is often a desired aspect of refining vegetable oils for their subsequent applications.

Refined vegetable oils have preferred colour, taste, and chemical stability for the consumer. Adsorbent materials such as clay minerals are typically used when refining vegetable oils. These remove low concentration components such as pigments, metals and lipid-oxides by adsorption and subsequent filtration. The removal of coloured components is where the term 'bleaching' originates from. Acid activated clays (AACs) such as montmorillonite are typically used. Montmorillonite is a layered hydrous aluminosilicate mineral with platelet-shaped particles.

Acid activation attacks and partly dissolves the layered mineral structure, leaving a partially amorphous material with a higher surface area and porosity. Surface acidity is also greatly enhanced, with more Brønsted and Lewis acid sites present.

Efficient removal of  $\beta$ -carotene from vegetable oils relies on adsorption to acid-activated clay. Despite this, studies detailing the interaction between  $\beta$ -carotene and AAC have given mixed results, with some indicating that physisorption occurs and others indicating chemisorption (Sabah, Çinar, & Çelik, 2007; N. Sarier & Güler, 1988; Nihal Sarier & Guler, 1989). Sarier *et al.* indicated adsorption to be chemical, through interaction with Brønsted and Lewis sites. They also suggested that the clay could be acting as an oxidation catalyst. Work by Silva *et al.* comparing the activity of AAC to untreated clay supported this observation. Measurements of peroxide and *p*-anisidine values indicated that the acid-activated clay was acting as an oxidiser, first producing primary oxidation products and then degrading them to secondary oxidation products (Silva *et al.*, 2014).

Notably, Sarier *et al.* also observed a colour change of the clay from white to blue upon  $\beta$ -carotene adsorption. All carotenoids are yellow, orange, or red in colour. However, systems in which carotenoids exhibit a blue colour do exist. In nature, the shells of many crustaceans such as lobster and sea snails have a blue-purple colour. This is due to the protein  $\beta$ -crustacyanin. Here, the carotenoid astaxanthin is bound to specific amino acid residues in a "carotenoprotein." Treatment with an organic solvent or heating releases the carotenoid with an accompanying colour change to back to orange (Helliwell *et al.*, 2002; Kildahl-

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Andersen, Frode Lutnaes, & Liaaen-Jensen, 2004).

Spectroscopic work carried out into carotenoid radicals adsorbed to silica-alumina materials has provided evidence that  $\beta$ -carotene and other carotenoids are oxidised upon adsorption. EPR and ENDOR studies showed that an electron is transferred from the carotenoid to  $Al^{3+}$  species on the surface of the adsorbent (A. L. Focsan, Magyar, & Kispert, 2015; A. Ligia Focsan et al., 2008; A. Ligia Focsan, Bowman, Molnár, Deli, & Kispert, 2011; A. Ligia Focsan & Kispert, 2017; Konovalova, Dikanov, Bowman, & Kispert, 2001; Konovalova, Gao, et al., 2001; Konovalova, Kispert, & Konovalov, 1997; Konovalov & Kispert, 1998). This work also noted that the presence of water hindered stable carotenoid radical formation on the adsorbent surface, indicating that water was involved in degradation of the adsorbed carotenoid species (Jeevarajan, Kispert, & Piekara-Sady, 1993).

The purpose of this study was to investigate the chemisorption of  $\beta$ -carotene to a commercially available AAC comparable to those used for vegetable oil bleaching (K10 montmorillonite). This mineral is well characterised with its mineralogical composition and structure already described in other work (Shimizu et al., 2008; Varadwaj, Rana, & Parida, 2013; Varadwaj, Rana, Parida, & Nayak, 2014; Wang et al., 2015). The AAC was treated with  $\beta$ -carotene with the resulting blue  $\beta$ -carotene-clay material (BC-AAC) then being isolated. This was extracted with solvents such as isopropanol (IPA) and tetrahydrofuran (THF). UPLC-MS/MS methods were developed to characterise the extracts and revealed a range of carotenoid oxidation products (COPs) to be present. An  $^{18}O$  labelled water exchange experiment showed the source of the oxygen atoms to be from water species also adsorbed to the clay surface. These findings provide insight into the removal mechanism of  $\beta$ -carotene and have implications for those wishing to efficiently decolourise vegetable oils in the food industry.

## 2. Materials and methods

### 2.1. Chemicals and materials

The solvents methanol, water, acetonitrile, dichloromethane (DCM) (all UPLC grade  $\geq 99.9\%$ ), IPA (Optima LC-MS grade), hexane (Chromasolv UPLC grade,  $\geq 99.5\%$ ) and toluene ((Chromasolv UPLC grade,  $99.9\%$ ) were acquired from Fisher Scientific (Loughborough, United Kingdom). The solvent additives formic acid (FA) and ammonium acetate were acquired from Fisher Chemical (Loughborough, United Kingdom).  $\beta$ -Carotene was acquired from Alfa Aesar ( $99.9\%$ , Fisher Scientific, Loughborough, United Kingdom).

THF (anhydrous, inhibitor free,  $\geq 99.9\%$ ), phosphoric acid, K10 montmorillonite and  $^{18}O$  labelled water (97 atom %, for PET) were acquired from Sigma-Aldrich (Gillingham, Dorset, United Kingdom).

### 2.2. Adsorption isotherm

The adsorption isotherm of  $\beta$ -carotene and AAC was measured using the depletion method, similar to that already reported (Wu & Li, 2009). The quantity of  $\beta$ -carotene adsorbed to the clay,  $Q_e$  (mg/g), was calculated using the following equation:

$$Q_e = \frac{V(C_0 - C_e)}{m} \quad (1)$$

where  $V$  (L), is the solution volume,  $C_0$  and  $C_e$  (mg/L) are the initial and equilibrium concentrations of  $\beta$ -carotene respectively, and  $m$  (g) is the mass of adsorbent. Portions of AAC (10 mg) were added to a series of vials in triplicate. Each had 10 mL  $\beta$ -carotene solution (of appropriately varied concentration) added to it. The vials were stirred for 1 h before the supernatant was collected by centrifugation and had its absorbance measured by UV-visible spectroscopy. The concentration of the supernatant was determined from the linear region of an absorbance/concentration plot of  $\beta$ -carotene. The adsorption isotherm data was fitted to

the equation for liquid phase Langmuir adsorption which is defined by the following equation:

$$\frac{C_e}{Q_e} = \frac{1}{q_m K_l} + \frac{C_e}{q_m} \quad (2)$$

where  $C_e$  and  $Q_e$  are as defined above,  $q_m$  (mg/g) is the maximum adsorbed amount, and  $K_l$  (L/mg) is the sorption equilibrium constant.

### 2.3. BC-AAC preparation

AAC (100 mg) was added to a suitable vessel together with  $\beta$ -carotene dissolved in DCM (10 mL,  $100 \text{ mg L}^{-1}$ ) and stirred at room temperature for 180 min. The appearance of the suspension rapidly became dark and cloudy. The solution was then separated from the BC-AAC by centrifugation, with the supernatant pipetted off.

The isolated BC-AAC was then stirred in a variety of solvents (hexane, toluene, THF, IPA and acetonitrile) in order to extract the adsorbate. The extract solution and clay were again separated by centrifugation, with the supernatant kept for UPLC-MS/MS analysis.

### 2.4. Direct infusion MS

Direct infusion mass spectra were recorded by nanospray ionisation on a Synapt G2S (Waters, Manchester, UK) fitted with a Triversa Nanomate chip-based nanospray system (Advion Biosciences, Norwich, UK). Samples were diluted by 20 times in methanol:DCM (50:50) and  $5 \mu\text{L}$  aspirated through a nanomate 400 chip by the nanospray robot.

### 2.5. UPLC-MS/MS analysis of extracts

UPLC-MS was performed on an Orbitrap Elite (Thermo Scientific, Hemel Hempstead, UK) equipped with a Dionex Ultimate 3000RS UPLC system. The column used was an Acquity BEH C18  $1.7 \mu\text{m}$  particle size,  $2.1 \times 150 \text{ mm}$  (Waters, Elstree, UK).

Solvent A was water/methanol (v:v, 1:1), and solvent B was IPA. Both solvents contained phosphoric acid ( $8 \mu\text{M}$ ), ammonium acetate ( $10 \text{ mM}$ ) and formic acid ( $0.1\% \text{ vol.}$ ). Flow rate was  $0.3 \text{ mL min}^{-1}$  and the sample injection volume was  $5 \mu\text{L}$ . The solvent composition was held at 41% B for 30 min, then increasing to 80% B by 31 min. This was held constant for 5 min, before returning to 41% B by 37.1 min and staying constant until 40 min. The MS spectra were acquired in positive ion electrospray ionisation mode at 120,000 resolution with an  $m/z$  range of 100–800. MS/MS spectra were acquired using collision induced dissociation (CID) fragmentation, with a collision energy of 35 eV, a resolution of 15,000 and an  $m/z$  range of 145–600.

### 2.6. $^{18}O$ labelled water exchange experiment

To exchange the water normally present on AAC clay surface with  $^{18}O$  labelled water, a portion of AAC was first dried to constant weight in a heated vacuum oven. The dried AAC was then stirred in  $^{18}O$  labelled water ( $0.5 \text{ mL}$ ) for 10 min. The slurry of  $^{18}O$  labelled water exchanged clay was then gently dried back to its original weight under ambient condition. This  $^{18}O$  labelled water exchanged clay was then used to prepare the carotenoid-clay materials in the same manner as described in section 2.3.

## 3. Results and discussion

### 3.1. Observations on $\beta$ -carotene adsorption

Upon mixing the  $\beta$ -carotene solution with the AAC particles the intensity of the orange solution rapidly diminished. The suspension developed a dark colour. After isolation from the suspension, the clay particles showed a dark blue colour as can be seen in Fig. 1. This

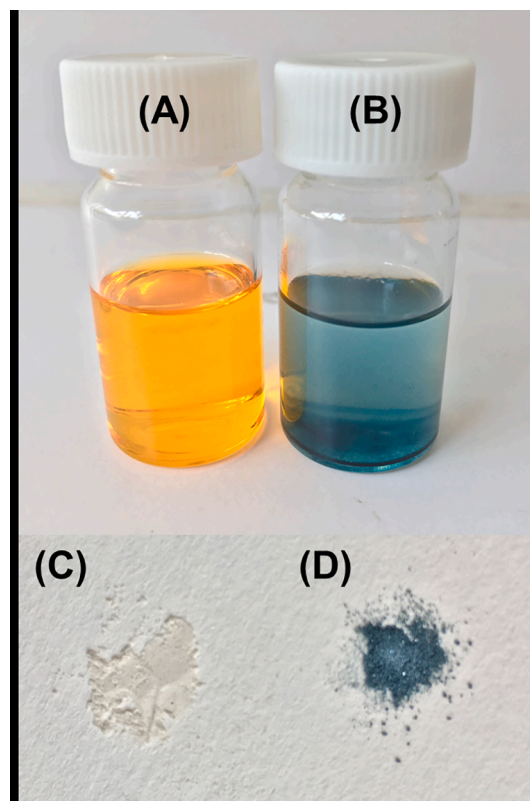


Fig. 1. Appearance of  $\beta$ -carotene solution (a),  $\beta$ -carotene solution after contact with AAC (b) and AAC before (c) and after adsorption of  $\beta$ -carotene (d).

suggested that  $\beta$ -carotene adsorption involves a sufficiently strong interaction to induce a hypsochromic shift similar to that observed in model systems of carotenoproteins (Helliwell et al., 2002; Kildahl-Andersen et al., 2004).

Subsequent suspension of the blue clay- $\beta$ -carotene particles in solvents such as THF or IPA gave solutions of an intense orange colour, suggesting surface compounds had been extracted. Suspension in less polar solvents such as hexane or toluene gave solutions with little or no colour. This indicated that surface compounds were relatively polar. This observation contrasts with that of Sarier et al., who suggested that surface compounds could not be extracted (Nihal Sarier & Guler, 1989). Direct infusion mass spectra of these extracts showed presence of multiple compounds of higher  $m/z$  than  $\beta$ -carotene. These were suspected to be oxygen containing carotenoids and this led to a more detailed accurate mass UPLC-MS investigation.

It was also observed that when IPA was used as the extraction solvent, the direct infusion mass spectra of the resulting samples contained extra peaks compared to the THF extracts (data not shown). These extra peaks had masses that suggested an additional reaction with IPA had occurred during the extraction. Swapping the solvent for BuOH showed an analogous set of peaks shift by  $m/z$  14 (when compared to IPA). Whilst interesting in themselves, these extra peaks made the evaluation of the oxidation products of the  $\beta$ -carotene-AAC interaction more cumbersome and so other solvents were employed that showed no reaction. Therefore, THF was chosen as the solvent for further extraction of surface compounds. UPLC-MS/MS was then used to characterise the THF extracts to better understand the chemistry occurring between  $\beta$ -carotene and AAC. It is suggested that protic solvents such as alcohols can react with the adsorbed carotenoid species either prior to or during the extraction process.

### 3.2. Adsorption isotherm

The adsorption isotherm of  $\beta$ -carotene to AAC was measured with the resulting plot visible in the supplementary information in Fig. S2. The data were fitted to the Langmuir and Freundlich models of adsorption. The data fitted the Langmuir model more closely than the Freundlich model, with the Langmuir plot being visible in the supplementary information in Fig. S3. It showed good linearity with  $R^2 = 0.997$ . Langmuir-type adsorption of  $\beta$ -carotene to AAC in organic solvents agrees with work by Khoo et al., Sarier et al., and Wu et al. (Wu & Li, 2009). However, Christidis et al. found that the adsorption of  $\beta$ -carotene in real oils more closely matched the Freundlich isotherm. This deviation from Langmuir behaviour could reflect the complexity of real oils. In the former case, a  $\beta$ -carotene molecule adsorbing to a clay surface is likely to only have other  $\beta$ -carotene molecules in its vicinity. However, in real oils there is likely to be a variety of nearby adsorbed molecules. The scope for more adsorbate-adsorbate interactions may account for the deviation from Langmuir-type adsorption to adsorption behaviour which fits the Freundlich isotherm (Christidis & Kosiari, 2003a). These points are considered further in section 3.6.

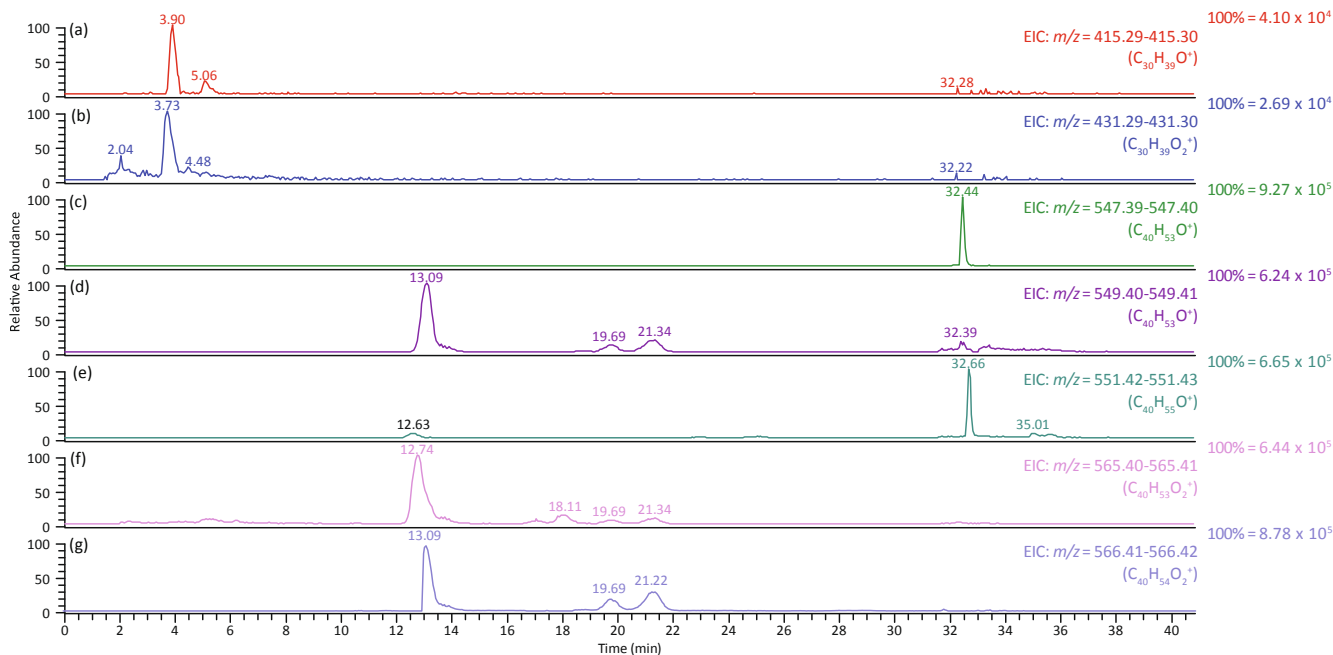
Fitting the data to the Langmuir model allowed the calculation of the maximum adsorbed amount ( $q_m = 65.8$  mg/g). This corresponds to an initial concentration of 80 mg/L of  $\beta$ -carotene. Coating the particles with a monolayer provides the maximum amount of surface compounds for extraction. This helped provide more intense mass spectra for analysis of the oxidation products. Subsequent BC-AAC particles were therefore prepared with an initial concentration corresponding to monolayer coverage.

### 3.3. UPLC-MS/MS on $\beta$ -carotene-AAC extracts

UPLC-MS/MS was used to characterise the compounds present in the extracts and therefore gain insight into the oxidation reaction between  $\beta$ -carotene and AAC. Many carotenoid chemical standards are expensive or difficult to acquire. Canthaxanthin was the only carotenoid standard that could be purchased for this investigation. Due to this, development of the UPLC-MS method was carried out using the extract samples. The method aimed to separate all major COPs by chromatographic and/or mass resolution. The MS/MS method aimed to provide tandem mass spectra for the major analytes to assist with tentative structural identification in the absence of satisfactory chemical standards.

UPLC-MS/MS data revealed that interaction with AAC produces a range of  $C_{40}$  carotenoids as well as many apocarotenoids produced by the scission of  $\beta$ -carotene. Fig. 2 shows high resolution extracted-ion-chromatograms (EICs) for 7 of the most abundant COP formulae. Multiple isomers are present in EICs for most formulae except those in (c) and (e) where one isomer is present. In each case one isomer appears to be the major COP. The current UPLC-MS/MS method allows resolution for the majority of detected COPs by either retention time and/or high resolution EIC in 40 min. The  $MH^+$  ions of the detected carotenoids were predominant except for the carotenoid with molecular formula  $C_{40}H_{54}O$ , where the  $M^+$  was predominant. This fell in line with expectations based on studies using acidified solvents for ESI-MS of polyenes (Guaratini, Lopes, Pinto, Colepicolo, & Gates, 2006; Guaratini et al., 2004). Most chromatographic peaks were resolved and had a satisfactory shape. Tailing was observed for some analytes, this was attributed to injection solvent strength as this was reduced by smaller injection volumes and worsened by larger injection volumes.

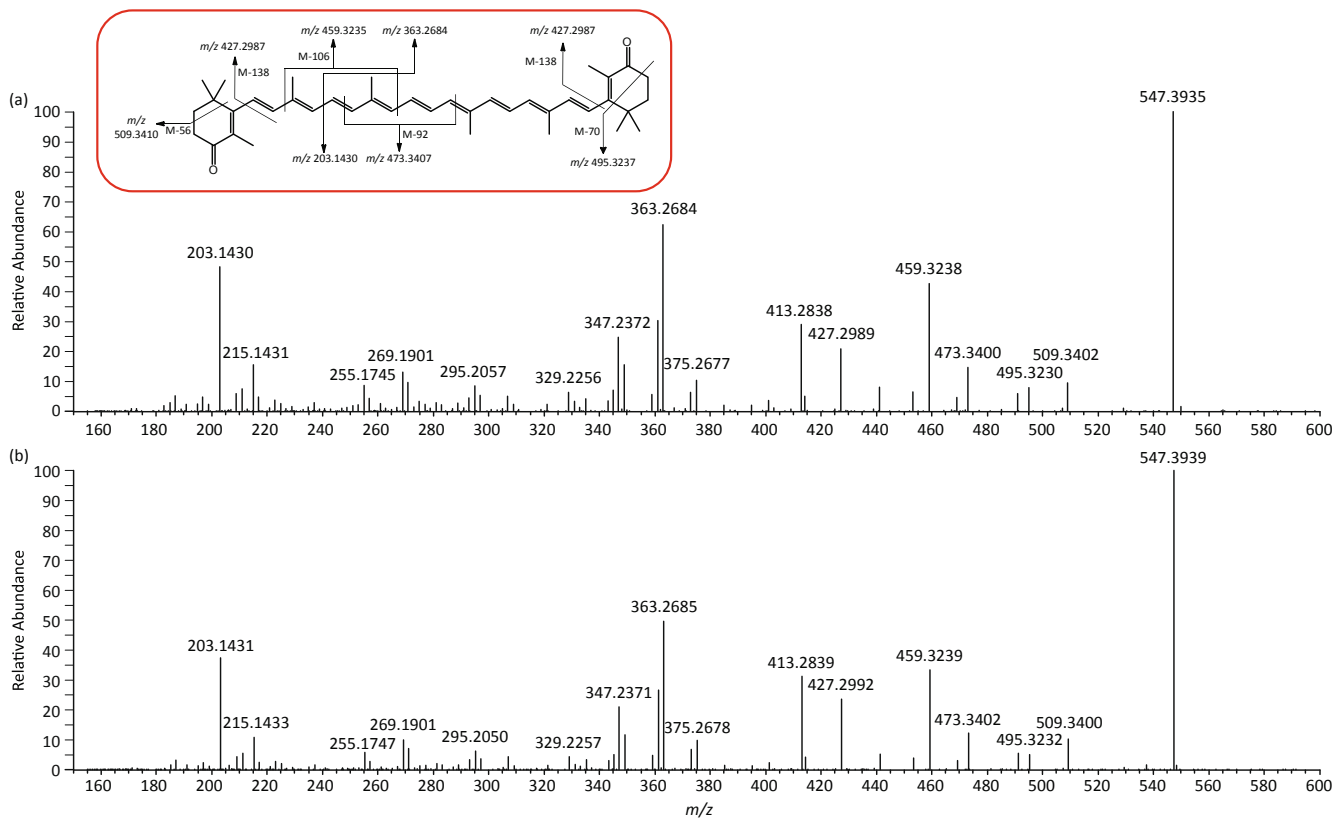
Most of the major COPs were  $C_{40}$  compounds with their formulae shown on the EICs in Fig. 2. A table summarising these major COPs can be found in Table S1 in the supplementary information. These carotenoids varied in the relative number of oxygen and hydrogen atoms, with all having more oxygen atoms and fewer hydrogen atoms than  $\beta$ -carotene ( $C_{40}H_{56}$ ). This indicates that the chemistry occurring on the surface of AAC can result in removal of hydrogen as well as the addition of oxygen. Notably, no  $\beta$ -carotene was detected in the extracts.



**Fig. 2.** Extracted-ion-chromatograms of the major carotenoid oxidation products. (a) red ( $C_{30}H_{38}O$ ), (b) blue ( $C_{30}H_{38}O_2$ ), (c) green ( $C_{40}H_{50}O$ ), (d) purple ( $C_{40}H_{52}O$ ), (e) teal ( $C_{40}H_{54}O$ ), (f) pink ( $C_{40}H_{52}O_2$ ) and (g) mauve ( $C_{40}H_{54}O_2$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A range of apocarotenoids and other short chain COPs produced from the scission of  $C_{40}$  carotenoids were also detected but typically had a lower abundance than the major  $C_{40}$  COPs. A complete table of detected apocarotenoids can be found in Table S2 the [supplementary](#)

[information](#). Of the apocarotenoids present, many had molecular formulae matching known COPs of  $\beta$ -carotene produced by enzymatic transformations and reaction with free radical oxygen. (Arathi, Sowmya, Vijay, Baskaran, & Lakshminarayana, 2015; Eroglu et al., 2012;



**Fig. 3.** Comparison of MS/MS spectra of the canthaxanthin standard (a) with the analyte suspected to be canthaxanthin (b). The precursor ion was the  $[M + H]^+$  at  $m/z$  565 in both cases. The fragmentation scheme is shown in the boxed insert.



Harrison, Dela Sena, Eroglu, & Fleshman, 2012; Mordi, Ademosun, Ajanaku, Olanrewaju, & Walton, 2020; Tang, Wang, Russell, & Krinsky, 1991). Notably  $\beta$ -apo-8-carotenal ( $C_{30}H_{40}O$ ), a typical COP, was not detected. However, two more unsaturated analogues with formulae  $C_{30}H_{38}O$  were detected. The EICs for compounds of this formula and their doubly oxygenated analogue ( $C_{30}H_{38}O_2$ ) are visible in Fig. 2 (a) and (b). Due to similarity in molecular formula and tentative MS/MS spectra interpretation (see section 3.5) it is suggested these compounds are dihydrogenated analogues of apocarotenal. However, it is difficult to confirm this in the absence of standards for these compounds. The observation of more unsaturated analogues of typical COPs was present throughout all detected carotenoids. For example, a compound with formula  $C_{10}H_{14}O$  was detected along with  $\beta$ -cyclocitral and  $\beta$ -cyclogeranic acid. Identities for the other apocarotenoids were suggested. These were based on the molecular formulae matching those of known COPs, and any trends in retention times. Use of a chemical standard and interpretation of fragmentation patterns allowed identification of four of the major COPs.

### 3.4. Identification of canthaxanthin using a chemical standard

One readily available and inexpensive carotenoid chemical standard is canthaxanthin. Comparison of the standard's retention and fragmentation pattern with the sample confirmed the presence of canthaxanthin in BC-AAC extracts. Fig. 3 shows the fragmentation pattern for the canthaxanthin standard (a) and the analyte suspected to be canthaxanthin (b). The two spectra are nearly identical, with all major peaks matching in exact mass and relative intensity. The insert in spectra (a) shows peak assignments resulting from cleavages of the canthaxanthin molecule. Examining the EICs in Fig. 4 for the standard, the extract, and a co-injection of standard and extract provides further evidence that canthaxanthin is the most abundant oxidation product with formula  $C_{40}H_{52}O_2$ . The peaks match in retention time, with the co-injection

showing an increase in relative intensity between the analyte eluting at 12.7 min and the other  $C_{40}H_{52}O_2$  analytes.

### 3.5. Identification of carotenoids by ESI-MS/MS

Where chemical standards were not available, tentative identifications were made from interpretation of MS/MS fragmentation spectra. The analyte  $[M + H]^+$  with molecular formula  $C_{40}H_{53}O^+$  at retention time = 13.76 had its fragmentation pattern interpreted. This can be seen in Fig. 5. The analyte was assigned as 3',4'-didehydro- $\beta$ -caroten-4-one.

Peaks at  $m/z$  443 and 457 correspond to loss of toluene and xylene. These are known losses from carotenoids with intact polyene chains. The fragment with  $m/z$  203 is shared with the MS/MS spectrum of canthaxanthin, suggesting presence of the same structural motif. This is likely due to vinyl-allyl cleavage between C10-C11. (Guaratini et al., 2006; Neto et al., 2016). Losses of  $m/z$  56 and 70 are also observed. These correspond to  $C_4H_8$  and  $C_5H_{10}$  losses respectively and are likely to originate from the saturated portions of the rings on each side of the molecule. Apart from the peaks due to loss of toluene and xylene, the most intense peak due to the loss of a hydrocarbon fragment is at  $m/z$  427. This is a loss of  $C_9H_{14}$  (mass 122) and is suggested to be due to loss of the ring indicated in the insert in Fig. 5. Loss of the terminal rings by breaking of the C6-C7 bond is observed in other carotenoid studies (Rivera, Christou, & Canela-Garayoa, 2014). The analyte also shows an intense peak at  $m/z$  411, indicating loss of  $C_9H_{14}O$  (mass 138). This is likely due to loss of the oxygen-containing ring indicated in Fig. 5.

Two apocarotenoids were also identified by interpretation of their MS/MS spectra. These had the formulae of  $C_{30}H_{38}O$  (RT = 3.90 mins) and  $C_{30}H_{38}O_2$  (3.73 mins). Their fragmentation spectra can be observed in Figs. S4 and S5 respectively, with their tentative structures shown in Fig. S6. For the  $C_{30}H_{38}O_2$  analyte, losses of CO and  $H_2O$  were observed. Low intensity fragments with  $m/z$  335 and 373 are likely due to losses of  $C_5H_8CO$  (mass 96) and  $C_2H_6CO$  (mass 58), respectively. Loss of  $CO_2$  was

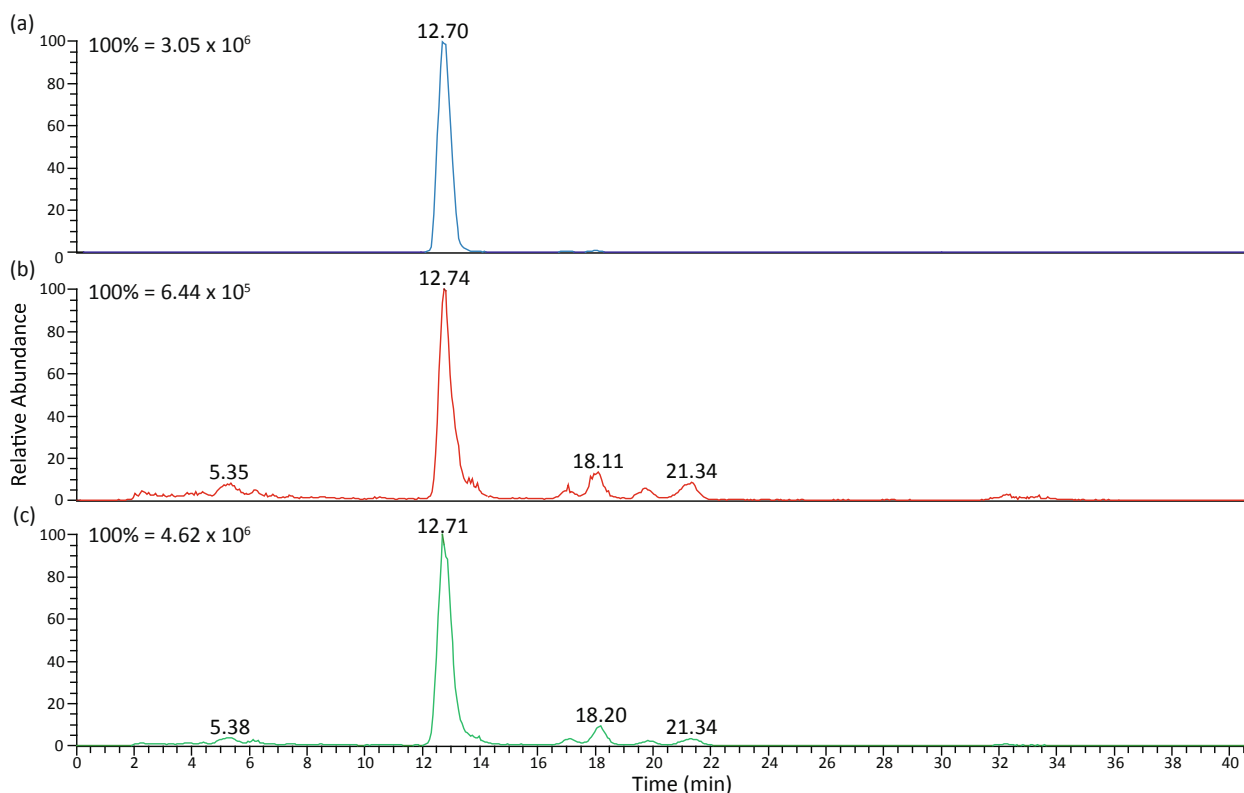


Fig. 4. Extracted Ion Chromatography (retention time: 0.00–40.84 min,  $m/z$  565.40–565.41 corresponding to the  $[M + H]^+$  of analyte with formula  $C_{40}H_{53}O_2^+$ ) of the canthaxanthin standard (a), the BC-AAB extract (b) and the BC-AAB and canthaxanthin standard co-injection (c).

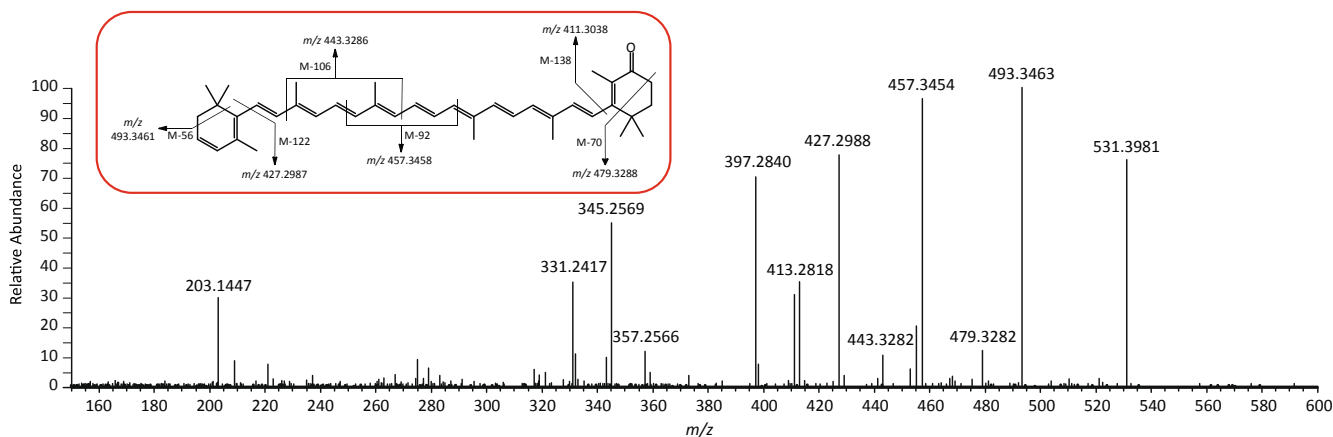


Fig. 5. MS/MS spectrum of carotenoid with  $m/z$  549.4088 and  $RT = 13.76$  mins, with proposed structure and fragmentation scheme shown in the boxed insert.

not observed. In addition, the presence of the fragment with  $m/z$  203 is common with canthaxanthin's fragmentation. This suggested that this analyte also possessed a keto group on the ring at position C4. Therefore, this analyte was tentatively assigned to be 4'-keto- $\beta$ -apo-8-carotenal. The analyte with formula  $C_{30}H_{38}O$  also showed losses corresponding to CO and  $H_2O$ . The presence of the fragment with  $m/z$  291 suggests loss of  $C_9H_{16}$  (mass 124), a terminal ring fragment the same as found in  $\beta$ -carotene. This, accompanied by the lack of peaks due to losses of  $C_5H_8CO$  and  $C_2H_6CO$  (as observed in the MS/MS of  $m/z$  431), indicate that the extra unsaturation is located in the polyene chain. Thus, this analyte was tentatively assigned to be 11',12'-didehydro- $\beta$ -apo-8-carotenal.

### 3.6. $^{18}O$ labelled water experiment

To better understand how  $\beta$ -carotene is oxidised upon interaction

with AAC, the source of the oxygen was sought. Clay minerals have water adsorbed to their surface under ambient conditions. This water was exchanged with  $^{18}O$  labelled water. The adsorption experiment was then carried out with the  $^{18}O$  labelled AAC.

Fig. 6 shows a comparison of the spectra of the oxygen containing carotenoids produced by reaction with normal AAC (a) and  $^{18}O$  labelled AAC (b). Spectrum (b) shows additional peaks, the accurate masses of which correspond to incorporation of one or two  $^{18}O$  atoms into the oxidised carotenoids produced by reaction with the  $^{18}O$  labelled AAC. The formula assignments for the  $^{18}O$  labelled compounds are summarised in Table S3 (see [supplementary information](#)). Remaining peaks for  $^{18}O$  carotenoids suggest that complete exchange of isotopically labelled water was not achieved.

Presence of the  $^{18}O$  labelled COPs shows that the adsorbed  $\beta$ -carotene molecules degrade by reaction with water present on the clay surface. This improves the understanding of the interaction between

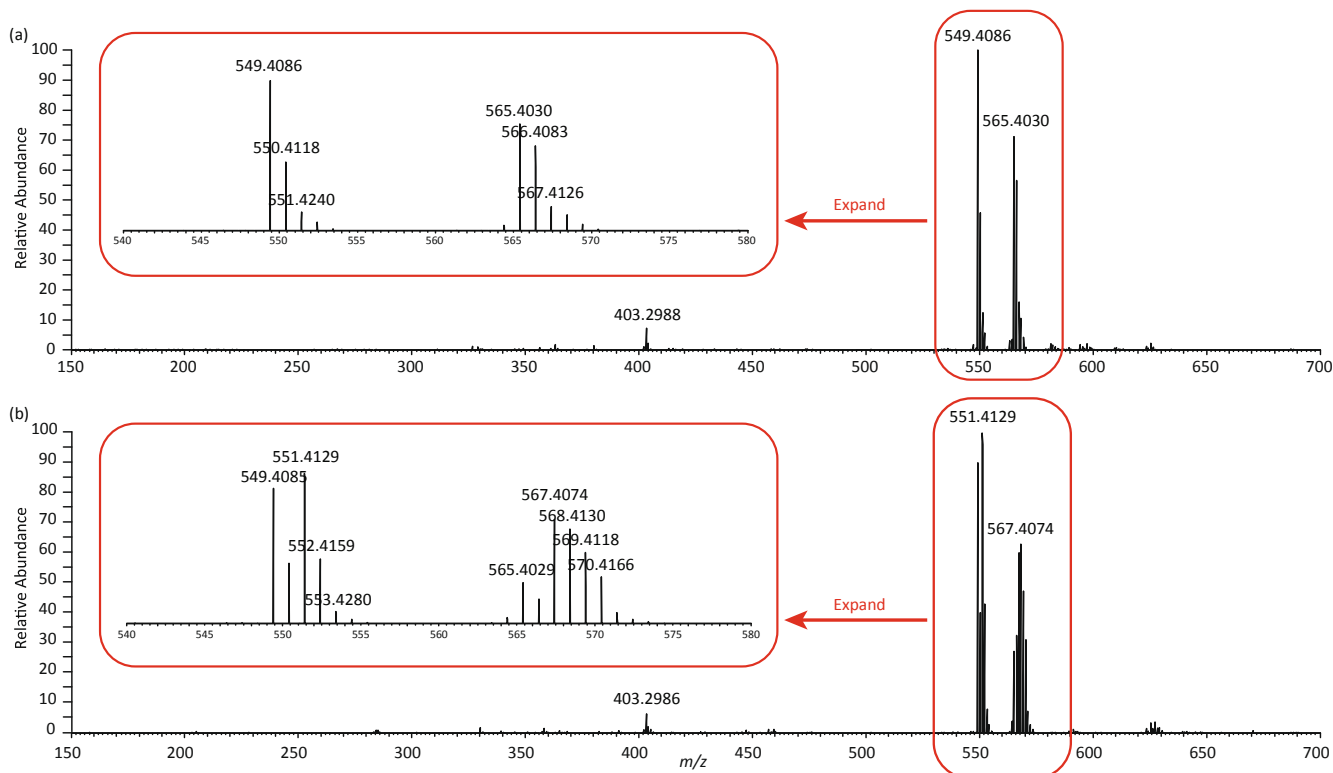


Fig. 6. Mass spectra with insert showing expansions of the isotopic ratios for oxidised carotenoids produced with normal clay (a) and  $^{18}O$  labelled clay (b).

AAC and  $\beta$ -carotene. Brønsted sites are the predominant type of acid site present on the surface of AAC. In addition, IR spectroscopy studies indicated that  $\beta$ -carotene interacts with the clay surface via these Brønsted sites (Nihal Sarier & Guler, 1989). Together, these findings provide extra mechanistic understanding. They indicate that the adsorption of  $\beta$ -carotene is due to interaction with Brønsted acid sites, with the resulting adsorbate undergoing reaction with adjacent water molecules. Interestingly, investigations into carotenoids adsorbed to silica-alumina materials showed that an electron is transferred from carotenoids to  $\text{Al}^{3+}$  Lewis acid sites (A. L. Focsan et al., 2015; A. L. Focsan & Kispert, 2017; Konovalova, Dikanov, et al., 2001; Konovalova & Kispert, 1998). This forms a radical cation which is also susceptible to hydrolysis by water molecules (Jeevarajan et al., 1993). Although there is scope for a similar process to occur with aluminium species exposed at the edges of AAC, this is likely to be much less significant than a process mediated by the more abundant Brønsted sites present in AACs. These data suggest that a certain moisture content in AAC adsorbents is necessary for successful  $\beta$ -carotene removal from vegetable oils.

In light of what has been discussed so far, these findings and their relation to the bleaching of real vegetable oils are considered further. As mentioned previously, Christidis et al. showed that the adsorption of  $\beta$ -carotene from maize and sunflower oils was characteristic of a Freundlich isotherm, indicating that the process may be heterogeneous. Work by Boki et al. also indicated that adsorption of  $\beta$ -carotene from a range of vegetable oils (rapeseed, soybean, wheatgerm, safflower, corn, cottonseed and sunflower) was also Freundlich in character (Boki, Kubo, Kawasaki, & Mori, 1992). However, Silva et al. determined that adsorption of  $\beta$ -carotene from palm oil was better fitted by the Langmuir model, though a good fit was still obtained by the Freundlich model. They also suggested this discrepancy could be due to difference in adsorbates (Silva et al., 2013). This argument explains these findings well. Palm oil has a high concentration of  $\beta$ -carotene. Whereas in the other oils  $\beta$ -carotene is not the principal pigment. In our work and that by Khoo et al., Sarier et al. and Wu et al.,  $\beta$ -carotene adsorption from organic solvents also gives rise to Langmuir style adsorption. Therefore, it is reasonable to suggest that the departure from Langmuir behaviour in real oils is due to the variety of adsorbates present. The prospect for competitive adsorption in vegetable oils is real, with adsorption sites likely occupied by a range of different molecules. This would decrease the potential for  $\beta$ -carotene degradation. However, the net effect is likely to be complicated, with Christidis et al. also describing processes facilitating transfer of pigment from the oil to the clay surface. Both Sarier et al. and Christidis et al. show evidence of a dual mechanism of adsorption. Where the initial step of chemisorption is followed by physisorption of those molecules to those adsorbed in the first step (Christidis & Kosiari, 2003; Nihal Sarier & Guler, 1989). While this may occur in systems where there is a large enough surplus of  $\beta$ -carotene in the continuous phase to coat the particles with a second layer, in this work the initial concentrations of carotene used approximately corresponded to a monolayer. Therefore, it is unlikely to occur in this instance. A logical next step of investigation would be an UPLC-MS<sup>2</sup> analysis of any COPs formed in the processing of real vegetable oils.

#### 4. Conclusions

An UPLC-MS/MS method was developed to resolve a complex mixture of carotenoid oxidation products produced from the interaction of  $\beta$ -carotene with an AAC typically used for bleaching vegetable oils. The analysis shows that the AAC oxidises  $\beta$ -carotene to a variety of carotenoids. C<sub>40</sub> carotenoids are produced in greater abundance than apocarotenoids produced from cleavage of the polyene chain. Some of the oxidation products are common to those produced via enzymatic pathways, such as  $\beta$ -apo-10-carotenal. However, the presence of many non-typical COPs shows the oxidation to be non-selective. Use of a chemical standard has confirmed canthaxanthin to be an abundant COP. ESI-MS/MS data has allowed the tentative identification of non-typical

COPs such as 3',4'-didehydro- $\beta$ -caroten-4-one. The production of unsaturated analogues of more common carotenoids indicates that the clay chemistry can remove hydrogen as well as add oxygen. An  $\text{H}_2^{18}\text{O}$  exchange experiment also showed that adsorbed water molecules are a key source of the oxygen in the reaction of  $\beta$ -carotene with AAC. These findings show that bleaching clay oxidises and degrades  $\beta$ -carotene, likely after interaction with surface Brønsted acid sites. They also provide insight into the mechanism of oxidation and underline the importance of considering adsorbent moisture levels when processing vegetable oils.

#### CRedit authorship contribution statement

**Andrew J. Hambly:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft. **Jeroen S. van Duijneldt:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing - review & editing. **Paul J. Gates:** Conceptualization, Formal analysis, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.129455>.

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